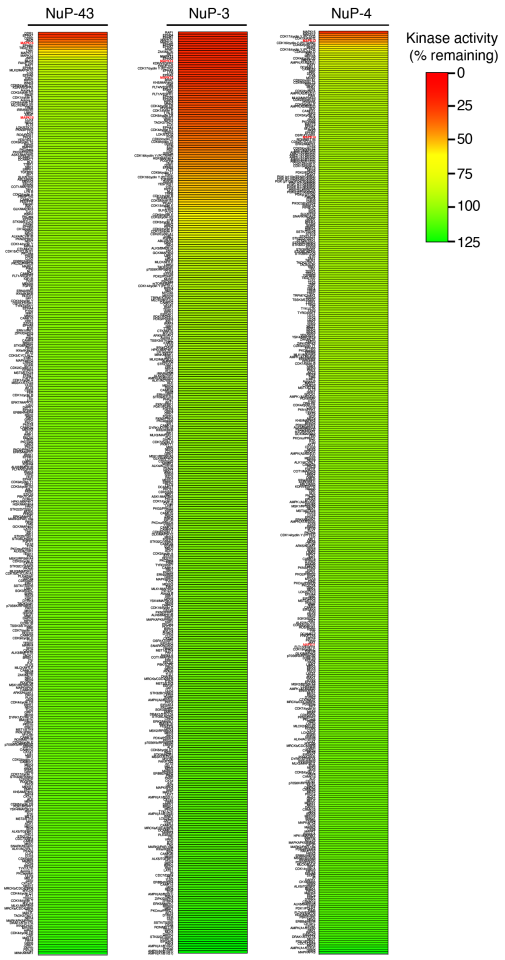
Supplemental Figures 1-10 and Tables 1-4

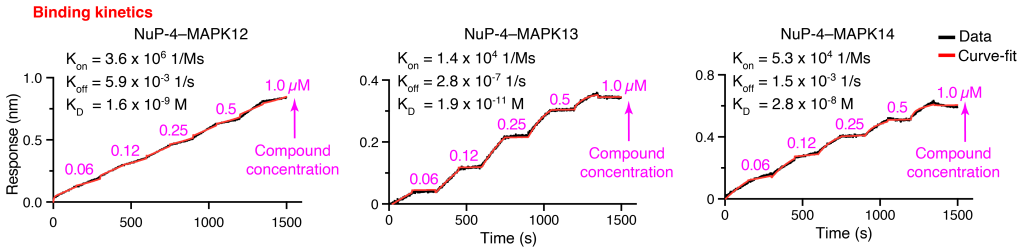
A first-in-kind MAPK13 inhibitor that modifies post-viral lung disease

Yong Zhang[1], Kangyun Wu[1], Dailing Mao[1], Courtney A. Iberg[1], Huiqing Yin-Declue[1], Kelly Sun[1], Hallie A. Wikfors[1], Shamus P. Keeler[1], Ming Li[1], Deanna Young[1], Jennifer Yantis[1], Erika C. Crouch[2], Joshua R. Chartock[1], Zhenfu Han[1], Derek E. Byers[1], Steven L. Brody[1], Arthur G. Romero[1], and Michael J. Holtzman[1,3,4*]

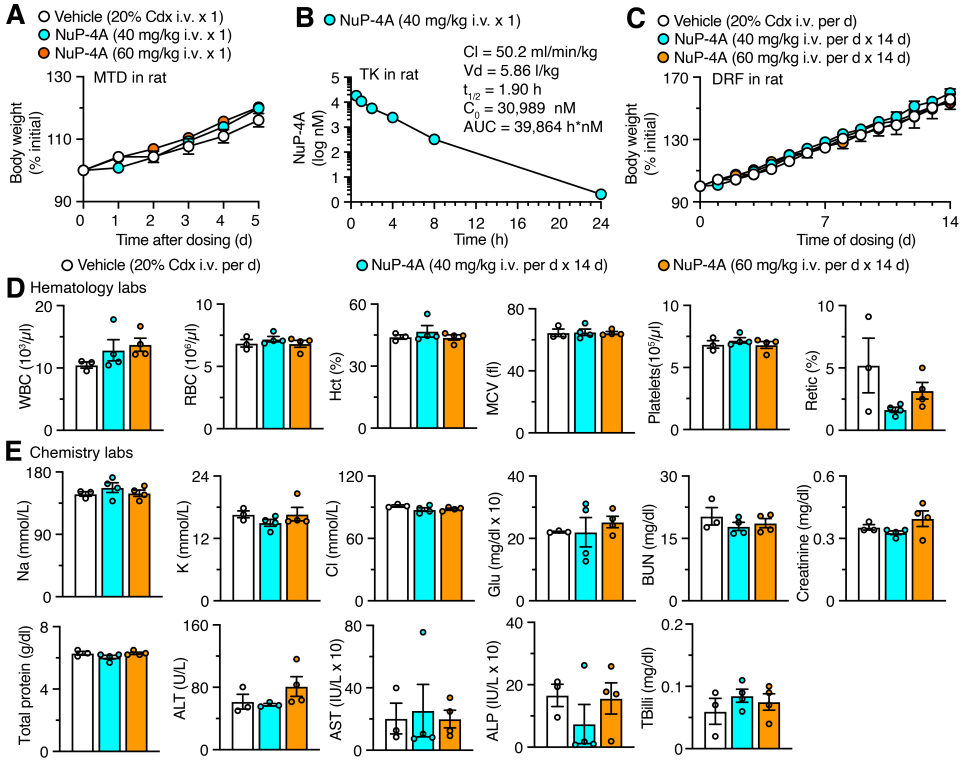
[1]Pulmonary and Critical Care Medicine, Department of Medicine, [2]Department of Pathology and Immunology, and [3]Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110 and [4]NuPeak Therapeutics Inc., St. Louis, MO 63105



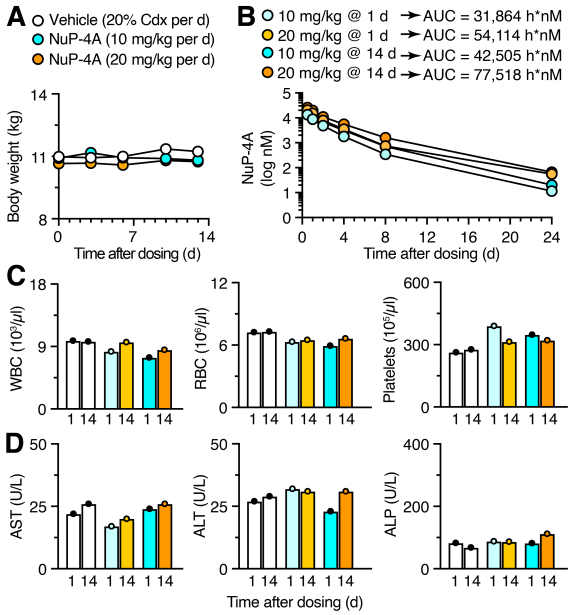
Supplemental Fig. 1. Kinase inhibition profile supports NuP-4 selectivity for MAPK13. Heat maps depict percentage of remaining kinase activity for NuP-43 (BIRB-796), NuP-3, and NuP-4 at 100 nM using a 425-kinase screening panel.



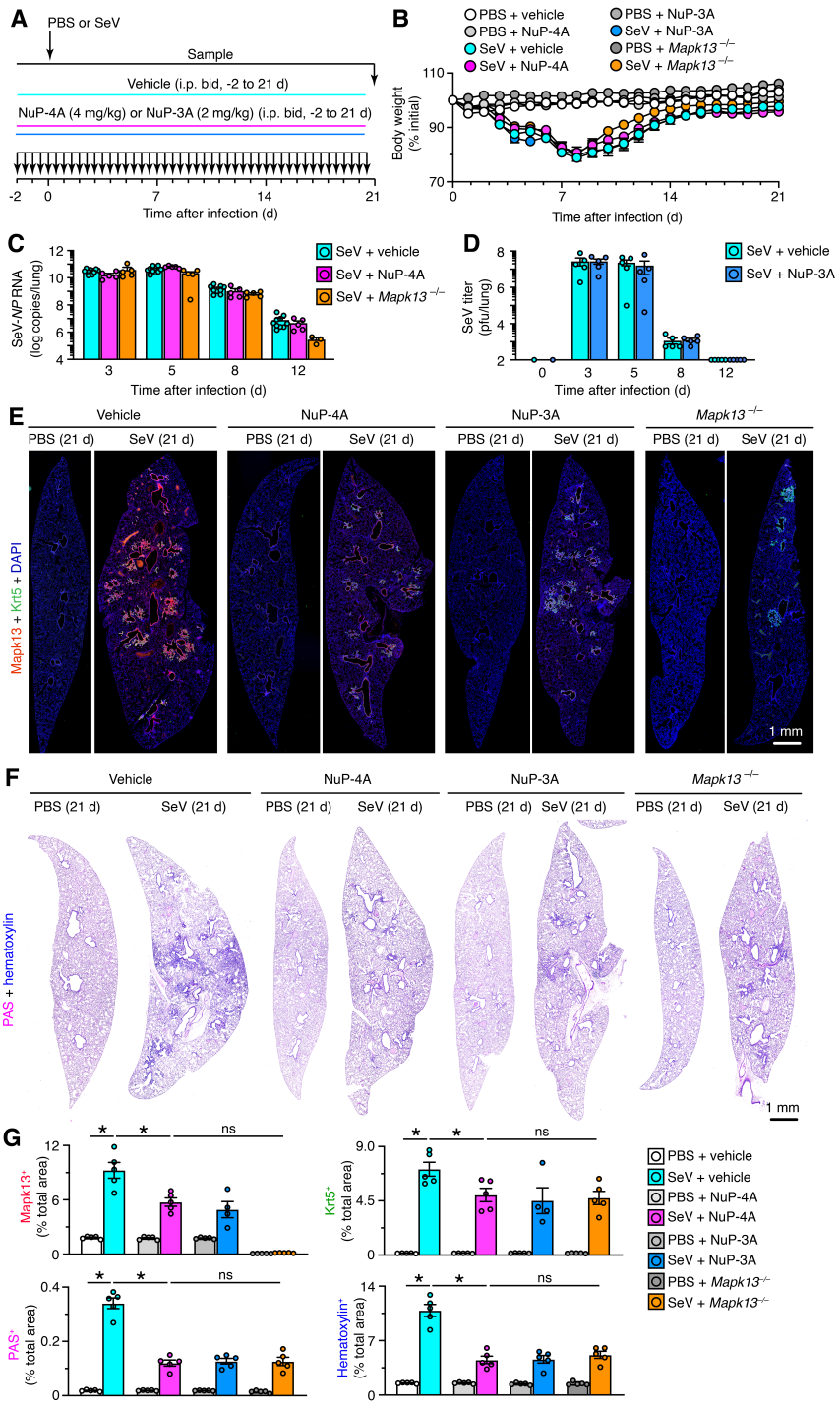
Supplemental Fig. 2. Binding kinetics support NuP-4 selectivity for MAPK13. Biolayer interferometry (BLI) analysis for NuP-4 binding to MAPK12, MAPK13, and MAPK14.



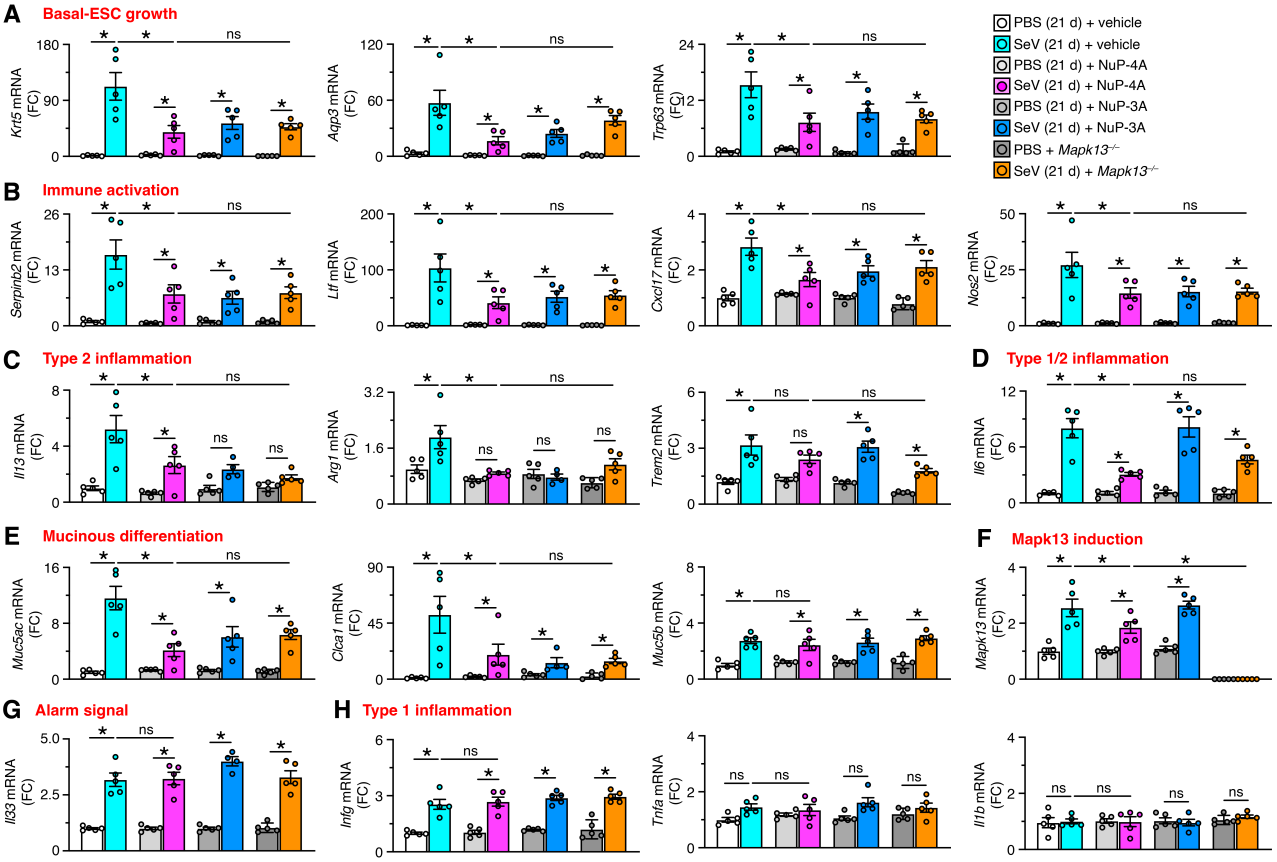
Supplemental Fig. 3. NuP-4 demonstrates safety in rat toxicology studies. A, Body weights from maximum tolerated (feasible) dose study limited to 60 mg/kg by compound solubility in excipient 2-hydroxypropyl-β-cyclodextrin (Cdx). **B**, Toxicokinetic (TK) analysis for NuP-4A with single dose at 40 mg/kg i.v. **C**, Body weights for dose-range finding (DRF) study at 40 and 60 mg/kg i.v. each day for 14 d. **D**, Hematology lab values for conditions in (c). **E**, Chemistry lab values for conditions in (C). Values represent mean ± s.e.m. (n=3-4 rats or mice per condition). No significant differences were detected from control vehicle (Cdx) condition using ANOVA and Tukey correction.



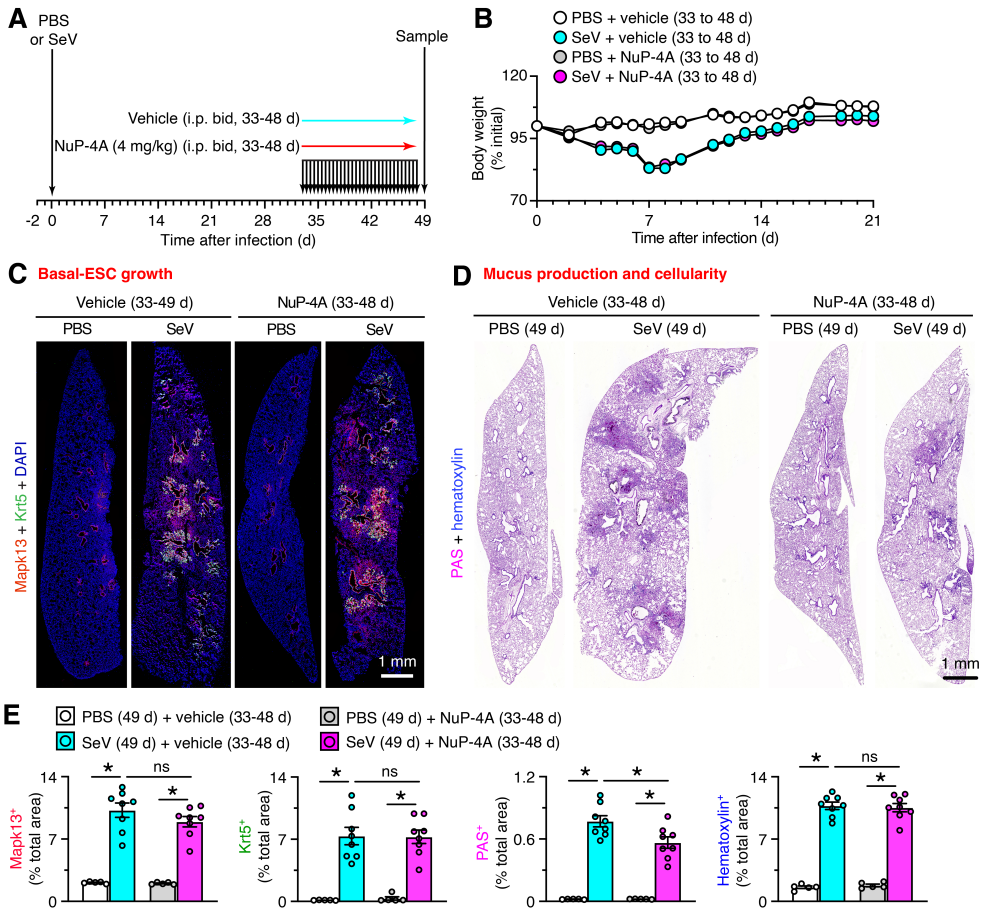
Supplemental Fig. 4. NuP-4 demonstrates safety in dog toxicology studies. A, Body weights for dose-range finding (DRF) study at 10 and 20 mg/kg i.v. each day for 14 d performed at maximum tolerated (feasible) dose study limited to 20 mg/kg by compound solubility. **B**, TK analysis for NuP-4A with single dose at 10 or 20 mg/kg i.v. at 1 and 14 d after starting the dose-range finding study. **C**, Hematology lab values for conditions in (B). **D**, Chemistry lab values for conditions in (B). Values represent mean ± s.e.m. (n=3-4 rats or mice per condition). No significant differences were detected from control vehicle condition using ANOVA and Tukey correction.



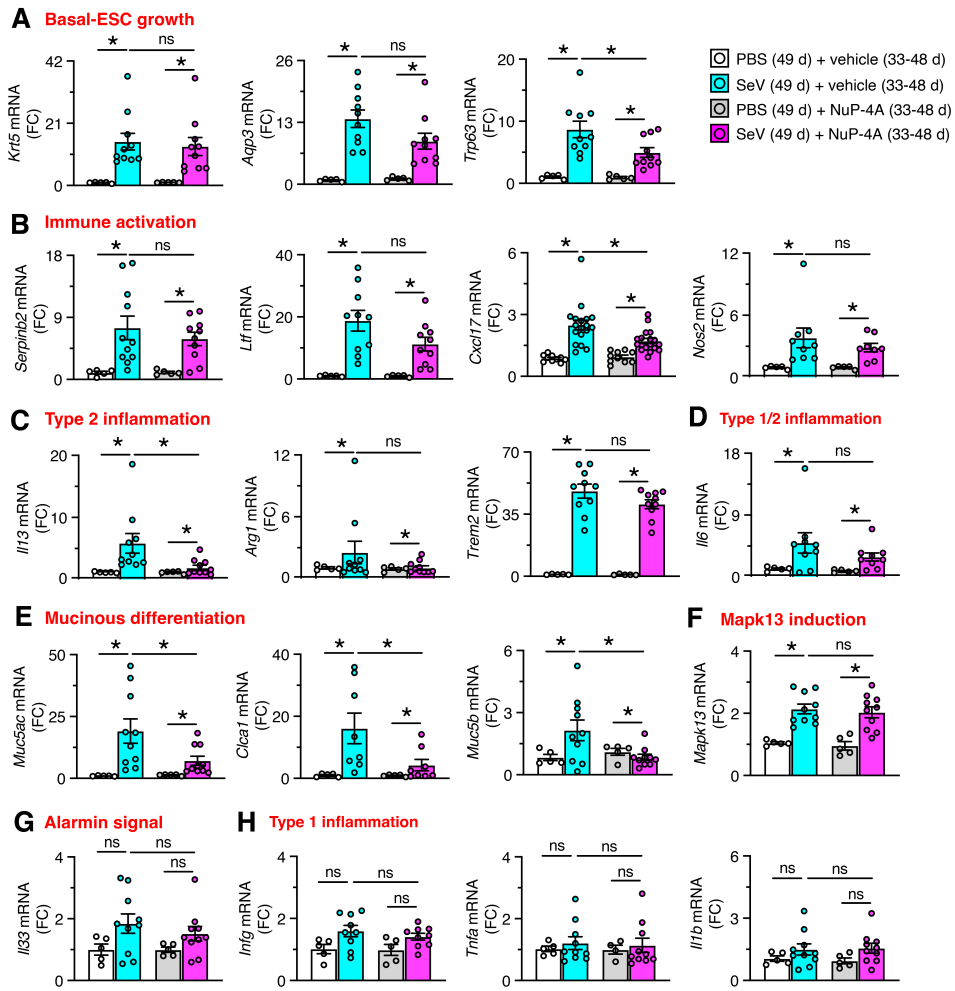
Supplemental Fig. 5. NuP-4A is equivalent to NuP-3A or Mapk13-deficiency in blocking PVLD at 21 d after infection in the mouse model based on histology. A, Protocol scheme for SeV infection and NuP-4A versus NuP-3A treatment or *Mapk13*-gene knockout and assessment during acute illness and 21 d after infection. **B**, Body weights for conditions in (A). **C,** Levels of viral RNA in lung tissue for NuP-4A treatment and *Mapk13*$^{-/-}$ conditions in (A). **D,** Corresponding levels of viral titer for NuP-3A treatment conditions in (A). **E**, Immunostaining for Mapk13 and Krt5 with DAPI counterstaining in lung sections for conditions in (A). **F**, PAS and hematoxylin staining of lung sections for conditions in (A). **G**, Quantitation of staining for conditions in (E,F). Data are representative of three separate experiments with n=8 animals per condition in each experiment (mean ± s.e.m.). **P* <0.05 using ANOVA and Tukey. correction. **P* <0.05 using ANOVA. Abbreviation: bid, twice daily; pfu, plaque-forming unit.



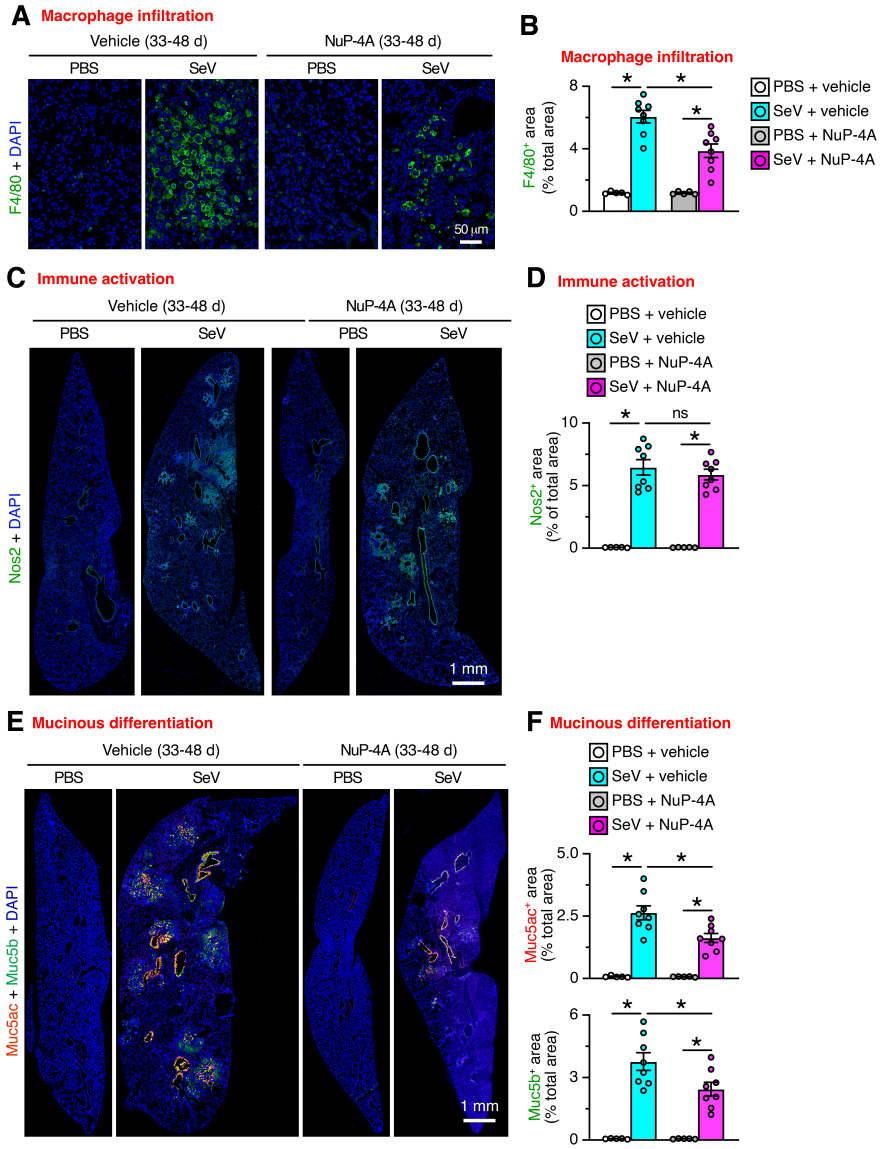
Supplemental Fig. 6. NuP-4A is equivalent to Mapk13-deficiency in blocking PVLD at 21 d after infection in the mouse model based on biomarkers. A-H, Lung levels of mRNA biomarkers for indicated disease endpoints for conditions in Supplemental Fig. 5A. Data are representative of three separate experiments with n=8 animals per condition in each experiment (mean ± s.e.m.). **P* <0.05 using ANOVA and Tukey. correction.



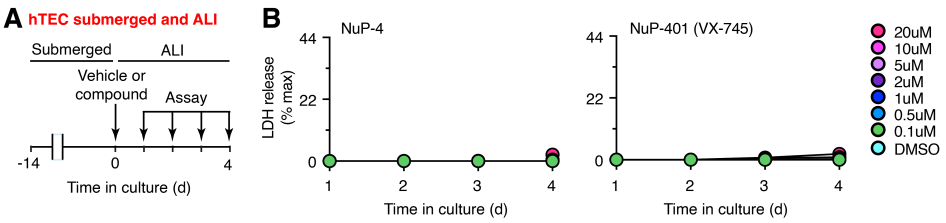
Supplemental Figure 7. NuP-4A reverses disease in the mouse model of PVLD. A, Protocol scheme for SeV infection or PBS control and then vehicle or NuP-4A treatment at 33-48 d after infection with assessment at 49 d after infection. **B**, Body weights for conditions in (A). **C**, Immunostaining for Mapk13 and Krt5 with DAPI counterstaining in lung sections for conditions in (A). **D**, PAS and hematoxylin staining of lung sections for conditions in (A). **E**, Quantitation of staining for conditions in (C,D). Data are representative of two separate experiments with n=8 animals per condition in each experiment (mean ± s.e.m.). *P <0.05 using ANOVA and Tukey correction.



Supplemental Figure 8. NuP-4A reverses biomarkers of type 2 inflammation and mucinous differentiation in the mouse model of PVLD. A-H, Lung levels of mRNA biomarkers for indicated disease endpoints for conditions in Fig. 7A. Data are representative of two separate experiments with n=8-16 animals per condition in each experiment (mean ± s.e.m.). *P <0.05 using ANOVA. *P <0.05 using ANOVA and Tukey. correction.



Supplemental Figure 9. NuP-4A reverses clinical biomarkers of macrophage infiltration and mucus production in the mouse model of PVLD. A. Immunostaining for F4/80 with DAPI counterstaining of lung sections for conditions in Fig. 7A. **B**, Quantitation of staining for (A). **C**, Immunostaining of lung sections for Nos2 with DAPI counterstaining for conditions in (A). **D**, Quantitation of immunostaining for (C). **E,** Immunostaining of lung sections for Muc5ac and Muc5b with DAPI counterstaining for conditions in (A). **F,** Quantitation of immunostaining for (E). Values represent mean ± s.e.m. (n=8 mice per condition). **P* <0.05 by ANOVA and Tukey correction.



Supplemental Fig. 10. NuP-4 is not cytotoxic in hTEC cultures. A, Protocol scheme for hTEC study using submerged and ALI culture conditions to assess cell toxicity with compound or vehicle control treatment using assay for LDH release. **B**, Levels of LDH release for conditions in (A). Values represent mean ± s.e.m. for a single subject representative of 3 subjects.

Supplemental Table 1. Sequences of DNA primers and probes for determining levels of SeV RNA in real-time qPCR assays.

Target gene	Type	Sequence
SeV-*NP*	F[1]	5'-GGCGGTGGTGCAATTGAG-3'
	R	5'-CATGAGCTTCTGTTTCTAGGTCGAT-3'
	P	5'-AGCTCTAGACAATGCC-3'

[1]Abbreviations: F, forward primer; R, reverse primer; P, MGB probe.

Supplemental Table 2. Antibodies for immunostaining mouse tissues and cells.

Target Protein	Antibody Type	Vendor	Catalogue #
F4/80	Rabbit mAb	Cell Signaling	70076
IL-33	Goat mAb	R&D Systems	AF3626
Ki-67	Mouse mAb	BD Pharmingen	550609
Ki-67	Rabbit mAb	Cell Signaling	12202
Krt5	Rabbit pAb	Abcam	ab53121
Mapk13	Rabbit pAb	R&D Systems	AF1519
Muc5ac	Mouse mAb (45M1), biotinylated	ThermoFisher	MS-145-B
Muc5ac	Mouse mAb (45M1)	ThermoFisher	MS-145-P
Muc5b	Rabbit pAb	Abcam	ab87276
Nos2	Rabbit pAb	Abcam	Ab3523
Sftpc	Rabbit pAb	Abcam	ab90716

[1]Abbreviations: mAb, monoclonal antibody, pAb, polyclonal antibody.

Supplemental Table 3. Sequences of DNA primers and probes for real-time qPCR assays in mouse tissue samples.

Target Gene	Type	ID/Sequence
Arg1		Mm00475988_m1 (ThermoFisher)
Aqp3		Mm.PT.58.13308206 (Integrated DNA Technologies)
Clca1	F[1]	5'-ACCGGCTGCCGCTAAAGAGCTTGAG-3'
	R	5'-AGACCATTGTTCTGAACCTGATCCGAAG-3'
	P	5'-AGCTGTCCAAAATGACAGGAGGCCTGCAGACATA-3'
Cxcl17		Mm.PT.58.28640067 (Integrated DNA Technologies
Gapdh		Mm.PT.39a.1 (Integrated DNA Technologies)
IFNg		Mm.PT.58.41769240 (Integrated DNA Technologies)
Il1b		Mm.PT.58.41616450 (Integrated DNA Technologies)
Il6		Mm.PT.58.10005566 (Integrated DNA Technologies)
Il13	F	5'-GGAGCTGAGCAACATCACACA-3'
	R	5'-CACACTCCATACCATGCTGCC-3'
	P	5'-CCAGACTCCCCTGTGCA-3'
Il33		Mm00505403_m1 (ThermoFisher)
Krt5		Mm.PT.58.41573083 (Integrated DNA Technologies)
Ltf		Mm00434787_m1 (ThermoFisher)
Mapk13	F	5'-GGAGCTACCCAAGACCTACCT-3'
	R	5'-TGTCCGCTTGTCGATGGCCGA-3'
	P	5'-GCGCACGTCGGCA-3'
Muc5ac	F	5'-TACCACTCCCTGCTTCTGCAGCGTGTCA-3'
	R	5'-ATAGTAACAGTGGCCATCAAGGTCTGTCT-3'
	P	5'-TATACCCCTTGGGATCCATCATCTACA-3'
Muc5b	F	5'-CTTTCACCCTCAGGAACACGAT-3'
	R	5'-TTCGAGGATTATACAGTTCAAAGCA-3'
	P	5'-TGAAGGACAAGGTGTGGAGATT-3'
Nos2		Mm.PT.58.43705194 (Integrated DNA Technologies)
SerpinB2		Mm.PT.58.13584177 (Integrated DNA Technologies)
Tnfa		Mm.PT.58.12575861 (Integrated DNA Technologies)
Trem2		Mm.PT.58.7992121 (Integrated DNA Technologies)
Trp63		Mm.PT.58.11081628 (Integrated DNA Technologies)

[1]Abbreviations: F, forward primer; R, reverse primer; P, MGB probe.

Supplemental Table 4. Sequences of DNA primers and probes for real-time qPCR assays in human cell samples.

Target Gene	Type	ID/Sequence
CXCL17	F	5'-GCTGCCACTAATGCTGATGT-3'
	R	5'-GAGCCATCTCCTAGAAGCCT-3'
	P	5'-CTCTGGCGACCCCTGGATTCAG -3
GAPDH	F	5'-CAGCCGAGCCACATCCCTCAGACACCAT-3
	R	5'-CTTTACCAGAGTTAAAAGCAGCCCTGGTGACCA-3'
	P	5'-AGGTCGGAGTCAACCGATTTGGTCGTATTG-3'
IL33	F	5'-AGACTTCTGGTTGCATGCC-3'
	R	5'-TCCAGGATCAGTCTTGCATTC-3'
	P	5'-CTGGTCTGGCAGTGGTTTTTCACAC -3'
MUC5AC	F	5'-AGGCCAGCTACCGGGCCGGCCAGACCAT-3'
	R	5'-GTCCCCGTACACGGCGCAGGTGGCCAGGCA-3
	P	5'-TGCAACACCTGCACCTGTGACAGCAGGAT-3

[1]Abbreviations: F, forward primer; R, reverse primer; P, MGB probe.